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# Immunohistochemical Staining for the p53 Protein and Proliferating Cell Nuclear Antigen in Familial Clustering of Gastric Cancer

YASUSHI OKUSA, MD, TAKASHI ICHIKURA, MD, AND SHOETSU TAMAKUMA, MD
*From the First Department of Surgery, National Defense Medical College, Tokorozawa, Japan*

Purpose of this study was to assess the role of p53 gene and tumor proliferating activity in familial clustering of gastric cancer.

Materials and Methods: Among 344 patients who underwent resections for gastric cancer, 10 patients had two or more gastric cancer-affected, first-degree relatives. We classified them as the group of gastric cancer with family history (FGC). Eighty-seven patients with gastric cancer who had no relatives with any malignant neoplasm were classified as the sporadic group. The paraffin-embedded specimens were stained immunohistochemically using monoclonal antibodies against the p53 product and proliferating cell nuclear antigen (PCNA).

Results: There was no significant difference in any clinicopathologic factor and the PCNA labeling index between the two groups. Staining for the p53 product was positive in 80% of the FGC group and in 38% of the sporadic group ($P < 0.05$).

Conclusion: Our study suggests that overexpression of p53 protein is one of the familial factors that correlates with carcinogenesis in the stomach. 

KEY WORDS: **p53 mutation, gastric cancer with family history, familial factor**

## INTRODUCTION

Epidemiologic studies on gastric cancer have reported that a family history of this disease is one of the possible risk factors [1], along with foods rich in nitrates or nitrites, a high salt diet, and smoking habits [2–4]. Mutations of the p53 gene have been reported to cause familial clustering of malignant neoplasms in the Li-Fraumeni syndrome, a hereditary disease characterized by the occurrence of diverse mesenchymal and epithelial neoplasms at multiple sites [5–7]. The p53 gene is believed to play an important role in the control of cell proliferation and tumor progression [8,9]. It is thought that the wild-type p53 gene product has a suppressor function on the growth of human colorectal carcinoma cells in vitro and in vivo and that the point mutations of this gene can abrogate its suppressive effect [10]. Although p53 expression has been studied in various human malignant neoplasms [11–27], there have been no published studies that have examined the relationship between p53 expression and familial clustering of gastrointestinal cancers. In this study, we investigated p53 expression and proliferating cell nuclear antigen (PCNA) expression in familial clustering of gastric cancer using immunohistochemical staining.

## MATERIALS AND METHODS

In order to identify those with a family history of gastric cancer, we examined the clinical records of 344 patients who underwent resections for gastric cancer between 1989 and 1994 at the First Department of Surgery, National Defense Medical College Hospital. Ten of the 344 patients (3%) had two or more first-degree relatives with gastric cancer, i.e., the proband's parents, children, or siblings. We classified these as the group of gastric cancer

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Address reprint requests to Dr. Yasushi Okusa, First Department of Surgery, National Defense Medical College, 3-2 Namiki, Tokorozawa 359, Japan.

with a family history (FGC). Among 238 patients with gastric cancer who had no relatives with any malignant neoplasms, there were 87 patients whose specimens were preserved during the same period as that of patients in the FGC group, and they were classified as the sporadic group. We excluded any patient whose specimen was fixed with formalin for 3 or more days since prolonged formalin fixation may decrease the antigenicity for the p53 protein or PCNA in immunohistochemical staining.

The tumor specimens that were fixed with a 10% neutralized formaldehyde solution were embedded routinely in paraffin. Each block was cut into 5-μm sections and deparaffinized in xylene and ethanol, followed by immersion in 3% hydrogen peroxide in 100% methanol and normal rabbit serum to inhibit endogenous peroxidase. The sections were stained using the streptavidin-biotin-peroxidase complex method (Histofine SAB-PO kit, Nichirei, Tokyo). Briefly, the sections were incubated with a 1:80 dilution of PAb1801, a mouse monoclonal antibody to the p53 product, which recognizes both the wild-type and mutant forms of the p53 protein (Oncogene Science, Mineola, NY). They were washed, then treated consecutively with a biotinylated rabbit antimouse antibody (Nichirei, Tokyo) and a streptavidin HRP-conjugated reagent (Nichirei, Tokyo) at room temperature. The bound peroxidase was visualized using a 0.05% solution of diaminobenzidine. The sections were counterstained with 2% methyl green and scored by two independent investigators who were blinded to the clinical data. The degree of staining was classified into four groups according to the percentage of tumor cell nuclei that stained positive: <5% as negative, 5–20% mild positive, 20–50% moderate positive, >50% strong positive. Negative controls for the immunostaining were carried out by replacing the primary antibody with PBS. As a positive control, we used sections from gastric adenocarcinoma previously shown to express high levels of p53.

Immunohistochemical staining for PCNA was performed in the same way as that for the p53 protein as described above using a monoclonal antibody, PC10 (Dakopatts, Denmark), at a dilution of 1:100. Cancer cells (500) were examined by light microscopy, and the percentage of cells with positively stained nuclei was expressed as the PCNA labeling index (LI).

The clinicopathologic findings were described according to the General Rules for Gastric Cancer Study proposed by the Japanese Research Society for Gastric Cancer (28). We tested the frequency distributions by the Chi-square test. The differences of the means of the continuous variables between the two groups were tested by Student's t-test. A $P$ value <0.05 was considered significant.

**TABLE I. Comparison of Clinicopathologic Features of FGC and Sporadic Groups**

| | FGC[a] (n = 10) | Sporadic (n = 87) |
|---|---|---|
| Sex | | |
| male : female | 6:4 | 58:29 |
| Age | 65.4 ± 7.5 | 59.6 ± 12.9 |
| Location of tumor | | |
| C[b] | 1 | 19 |
| M[c] | 5 | 34 |
| A[d] | 4 | 34 |
| Histologic classification | | |
| differentiated | 7 | 45 |
| undifferentiated | 3 | 42 |
| Depth of invasion | | |
| -mp[e] | 6 | 46 |
| ss-[f] | 4 | 41 |
| Lymph node metastasis | | |
| − | 6 | 42 |
| + | 4 | 45 |
| Lymphatic invasion | | |
| − | 3 | 27 |
| + | 7 | 60 |
| Venous invasion | | |
| − | 7 | 56 |
| + | 3 | 31 |

[a]Gastric cancer with family history.
[b]Cardia (upper third of stomach).
[c]Median third of stomach.
[d]Antrum (lower third of stomach).
[e]Muscularis propria or shallower.
[f]Subserosa or deeper.

## RESULTS

### Clinicopathologic Data

The clinicopathologic characteristics of those in the FGC and sporadic groups are listed in Table I. The FGC group consisted of six men and four women with an average age of 65.4 years. There were no significant differences between the groups in terms of gender, average age, tumor sites, depth of invasion, histologic classification, lymph node involvement, or vessel invasion.

### Expression of p53 Protein

The typical staining for p53 protein was demonstrated in Figure 1. The difference in staining for p53 protein between the FGC group and sporadic group was statistically significant ($P < 0.05$). In the FGC group, 8 of 10 cases (80%) showed mild to strong positive staining for p53 protein, whereas only 33 of the 87 cases (38%) in the sporadic group showed positive staining (Fig. 2). In the FGC group, there were six cases (60%) with moderate or strong positive staining in contrast with 27 cases (31%) in the sporadic group.

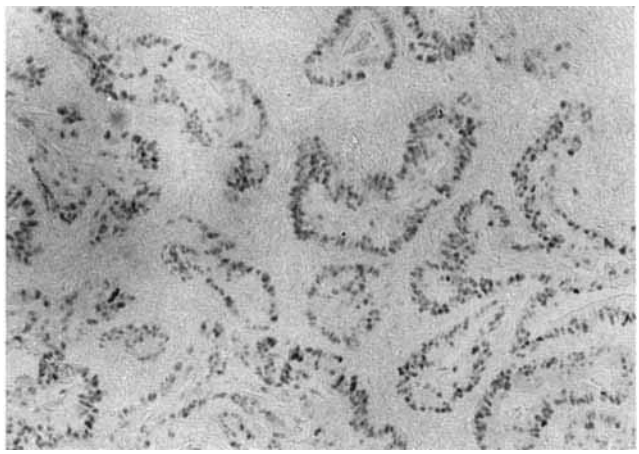

Fig. 1. Immunohistochemical staining of gastric cancer with a monoclonal antibody against p53 protein, PAb1801. Many cells are positive. A methyl green counterstain was used.

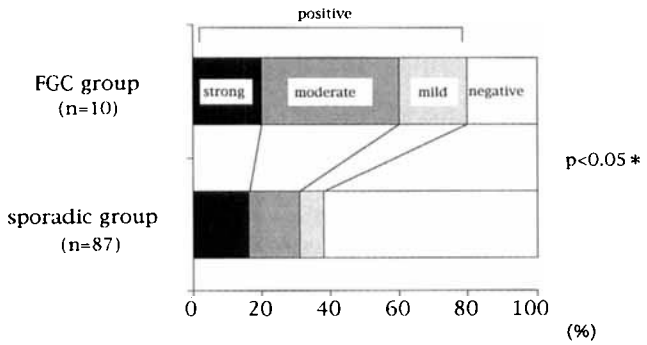


Fig. 2. Immunohistochemical staining for p53 protein. The staining for p53 was classified into four groups according to the percentage of nuclei of the tumor cells that stained positive: <5% as negative, 5–20% mild positive, 20–50% moderate positive, >50% strong positive. *Chi-square test.

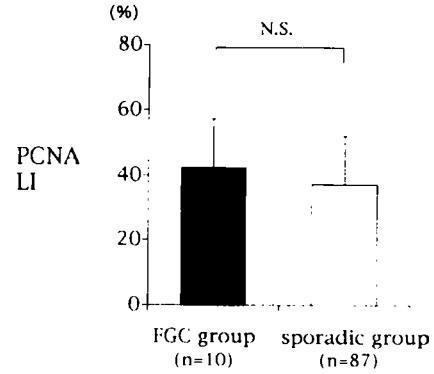


Fig. 3. PCNA labeling index (LI). PCNA, proliferating cell nuclear antigen; N.S., not significant ($P > 0.05$).

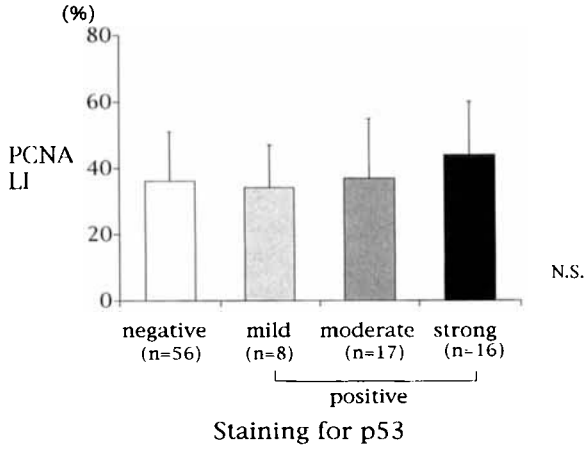


Fig. 4. Relationship between PCNA and p53. The staining for p53 was classified into four groups as described in the legend for Figure 2. PCNA, proliferating cell nuclear antigen; N.S., not significant ($P > 0.05$).

## PCNA Labeling Index (LI)

The average PCNA LI was 42 ± 15% (mean ± SD) in the FGC group and 37 ± 15% in the sporadic group, with no significant difference between the groups (Fig. 3).

There was no correlation between the PCNA LI and increased p53 expression. The average PCNA LI was 44 ± 16% in the 16 patients with >50% positive staining for p53 (3+), 37 ± 18% in the 17 with p53 (2+), 34 ± 13% in the 8 with p53 (1+), and 36 ± 15% in the 56 with negative staining (Fig. 4).

## DISCUSSION

The p53 gene, encoded on chromosome 17p, is believed to be an important negative regulator of cellular proliferation and malignant progression [10,29] although the molecular mechanisms of its action are not clearly understood. However, mutational inactivation of this gene seems to facilitate carcinogenesis. Most of the mutations alter the conformation of the nuclear protein product, which can inactivate any wild-type p53 protein present [30]. The half-life of the wild-type p53 gene product is short, whereas that of some mutant forms is prolonged [31]. Therefore, most of the protein detected by the immunohistochemical staining is a mutated form of the p53 gene product. In fact, the increased expression of the p53 protein in tumors and tumor cell lines has been reported to correlate with the presence of missense point mutations in the four evolutionarily conserved regions of the p53 gene [30,32,33]. The overexpression of p53 protein could be a result of forming complexes of wild-type p53 and other oncogenic proteins such as mdm2 gene product [34].

Elevated expression of the p53 protein, or mutational inactivation of the p53 gene, has been shown in various human malignant tumors, including carcinomas of the

breast, colon, and rectum, stomach, bladder, prostate, and head and neck [11–27]. Starzynska et al. [19] have reported that elevated expression of the p53 protein was associated with an advanced stage of the disease and unfavorable clinical outcome in gastric and colorectal carcinomas. Martin et al. [20] also have found a significant correlation between the level of p53 expression and survival time in gastric cancer. However, Purdie et al. [17] and Suzuki et al. [18] have found no significant correlation between the level of p53 expression and the stage of the disease or the clinicopathologic variables related to biologic aggressiveness in colorectal cancer.

Germ-line p53 mutations have been considered to cause the Li-Fraumeni syndrome, a dominantly inherited disorder with a high incidence of breast cancer, sarcomas, and other neoplasms, predisposing the family members to cancer [5,6]. Thor et al. [11] have found a significantly increased incidence of breast carcinomas with elevated expression of the p53 protein in patients with the Li-Fraumeni syndrome, familial breast cancer, or the familial breast and ovarian cancer syndrome. However, there are no published data on the relationship between the level of p53 expression and familial clustering of gastrointestinal cancer. In our study, a significantly higher incidence of positive staining for the p53 gene product was observed in the patients with familial clustering of gastric cancer than in those without familial clustering. We could not find any significant difference in the average age between the two groups, which contrasts with other malignant neoplasms associated with inherited genetic abnormalities. It remains to be determined which genetic or environmental factors lead to elevated expression of the p53 protein, shared by family members.

We found no correlation between the level of p53 expression and cellular proliferation as measured by immunohistochemical staining for PCNA. Wild-type p53 may inhibit cell-cycle progression into S-phase by down-regulation of PCNA mRNA [35]. Increased accumulation of p53 protein has been reported to be associated with increased cellular proliferation as detected by increased PCNA staining of cancers or severely dysplastic adenomas [22,35,36]. Barbareschi et al. [25] have found no relationship between altered p53 expression and the percentage of PCNA labeled cells in central nervous system neoplasms, which is consistent with our results. Visakorpi et al. [22] have reported that there were some p53-negative prostate cancers with a high S-phase fraction. The p53 protein may not be the only factor that can control PCNA gene expression.

Our study suggests that overexpression of p53 protein is one of the familial factors that correlates with carcinogenesis in the stomach. We believe that immunohistochemical staining for the p53 protein in cancer tissue could be of help in identifying families in which screening for early gastric cancer could be useful.

## REFERENCES


1. Hoshino H, Hirayama T, Arimoto H, et al.: Gastric cancer risk factors: A case-control study based on medical records. Jpn J Cancer Res 76:846–850, 1985.
2. Mirvish SS: The etiology of gastric cancer. Intragastric nitrosamide formation and other theories. J Natl Cancer Inst 71:629–647, 1983.
3. Hirayama T: Epidemiology of stomach cancer in Japan with special reference to the strategy for the primary prevention. Jpn J Clin Oncol 14:159–168, 1984.
4. Tajima K, Tominaga S: Dietary habits and gastrointestinal cancers: a comparative case-control study of stomach and large intestinal cancers in Nagoya, Japan. Jpn J Cancer Res 76:705–716, 1985.
5. Srivastava S, Zou ZQ, Pirollo K, et al.: Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature 348:747–749, 1990.
6. Malkin D, Li FP, Strong LC, et al.: Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 250:1233–1238, 1990.
7. Li FP, Fraumeni JF: Soft-tissue sarcomas, breast cancer, and other neoplasms: A familial syndrome? Ann Intern Med 71:747–752, 1969.
8. Hollstein M, Sidransky D, Vogelstein B, Harris CC: p53 mutations in human cancer. Science 253:49–53, 1991.
9. Baker SJ, Preisinger AC, Jessup JM, et al.: p53 gene mutations occur in combination with 17q allelic deletions as late events in colorectal tumorigenesis. Cancer Res 50:7717–7722, 1990.
10. Baker SJ, Markowitz S, Fearon ER, et al.: Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 2 49:912–915, 1990.
11. Thor AD, Moore II DH, Edgerton SM, et al.: Accumulation of p53 tumor suppressor gene protein: An independent marker of prognosis in breast cancers. J Natl Cancer Inst 84:845–855, 1992.
12. Iwaya K, Tsuda H, Hiraide H, et al.: Nuclear p53 immunoreaction associated with poor prognosis of breast cancer. Jpn J Cancer Res 82:835–840, 1991.
13. Moll UM, Riou G, Levine AJ: Two distinct mechanisms alter p53 in breast cancer: Mutation and nuclear exclusion. Proc Natl Acad Sci USA 89:7262–7266, 1992.
14. Cattoretti G, Rulke F, Andreola S, et al.: p53 expression in breast cancer. Int J Cancer 41:178–183, 1988.
15. Walker RA, Dearing SJ, Lane DP, Varley JM: Expression of p53 protein in infiltrating and in-situ breast carcinomas. J Pathol 165:203–211, 1991.
16. Ostrowski JL, Sawan A, Henry L, et al.: p53 expression in human breast cancer related to survival and prognostic factors: An immunohistochemical study. J Pathol 164:75–81, 1991.
17. Purdie CA, O'Grady J, Piris J, et al.: p53 expression in colorectal tumors. Am J Pathol 138:807–813, 1991.
18. Suzuki H, Matsumoto K, Koide A, et al.: Correlation of p53 with the clinicopathologic features and prognosis of colorectal adenocarcinoma. Jpn J Surg 24:85–87, 1994.
19. Starzynska T, Bromley M, Ghosh A, Stern PL: Prognostic significance of p53 overexpression in gastric and colorectal carcinoma. Br J Cancer 66:558–562, 1992.
20. Martin HM, Filipe MI, Morris RW, et al.: p53 expression and prognosis in gastric carcinoma. Int J Cancer 50:859–862, 1992.
21. Lipponen PK: Over-expression of p53 nuclear oncoprotein in transitional-cell bladder cancer and its prognostic value. Int J Cancer 53:365–370, 1993.
22. Visakorpi T, Kallioniemi OP, Heikkinen A, et al.: Small subgroup of aggressive, highly proliferative prostatic carcinomas defined by p53 accumulation. J Natl Cancer Inst 84:883–887, 1992.
23. Frank JL, Bur ME, Garb JL, et al.: p53 tumor suppressor oncogene expression in squamous cell carcinoma of the hypopharynx. Cancer 73:181–186, 1994.
24. Shin DM, Kim J, Ro JY, et al.: Activation of p53 gene expression in premalignant lesions during head and neck tumorigenesis. Cancer Res 54:321–326, 1994.
25. Barbareschi M, Iuzzolino P, Pennella A, et al.: p53 protein expression in central nervous system neoplasms. J Clin Pathol 45:583–586, 1992.
26. Frankel RH, Bayona W, Koslow M, Newcomb EW: p53 mutations

in human malignant gliomas: comparison of loss of heterozygosity with mutation frequency. Cancer Res 52:1427–1433, 1992.
27. Ellison DW, Gatter KC, Steart PV, et al.: Expression of the p53 protein in a spectrum of astrocytic tumours. J Pathol 168:383–386, 1992.
28. Japanese Research Society for Gastric Cancer: "Japanese Classification of Gastric Carcinoma." Tokyo: Kanehara, 1995.
29. Finlay CA, Hinds PW, Levine AJ: The p53 protooncogene can act as a suppressor of transformation. Cell 57:1083–1093, 1989.
30. Bartek J, Iggo R, Gannon J, et al.: Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene 5:893–899, 1990.
31. Finlay CA, Hinds PW, Tan TH, et al.: Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol Cell Biol 8:531–539, 1988.
32. Iggo R, Gatter K, Bartek J, et al.: Increased expression of mutant forms of p53 oncogene in primary lung cancer. Lancet 335:675–679, 1990.
33. Davidoff AM, Humphrey PA, Iglehart JD, Marks JR: Genetic basis for p53 overexpression in human breast cancer. Proc Natl Acad Sci USA 88:5006–5010, 1991.
34. Marchetti A, Buttitta F, Pellegrini S, et al.: Mdm2 gene amplification and overexpression in non-small cell lung carcinomas with accumulation of the p53 protein in the absence of p53 gene mutations. Diagn Mol Pathol 4:93–97, 1995.
35. Mercer WE, Shields MT, Lin D, et al.: Growth suppression induced by wild-type p53 protein is accompanied by selective down-regulation of proliferating-cell nuclear antigen expression. Proc Natl Acad Sci USA 88:1958–1962, 1991.
36. Pignatelli M, Stamp GWH, Kafiri G, et al.: Over-expression of p53 nuclear oncoprotein in colorectal adenomas. Int J Cancer 50:683–688, 1992.

## COMMENTARY

Gastric carcinoma prevalence has declined in recent decades, but it remains a highly lethal disease when allowed to progress beyond the earliest stages. Gastroscopic examination currently is the only method available to discover gastric carcinoma in its potentially curable presymptomatic phase. Prevalence levels generally do not justify costs of gastroscopic screening of the general population, but a small segment of the population with a higher prevalence rate could be screened if identified. The report in this issue by Okusa et al. describes an association between immunohistochemical identification of p53 in gastric carcinoma and familial occurrence of the disease. The authors suggest that p53 overexpression in their carcinomas can identify kindreds with familial gastric carcinoma and suggest screening of these kindreds for early stages of the disease.

The data presented by Okusa et al. show that in the population studied, the cutoff for immunohistochemical p53 positivity that they suggest—strong-intermediate vs. weak/negative—resulted in the following values: sensitivity 80%, specificity 62%, positive predictive value 23%, false positive rate 38%, and efficiency of classification 64%. This means that strong-intermediate p53 staining identified 80% of patients with familial gastric carcinoma while falsely identifying 38% as belonging to gastric carcinoma families when they did not. Of those identified as possibly belonging to gastric carcinoma kindreds, only ~20% would actually prove to belong to such a kindred. Nonetheless, at this rate individuals might be willing to undergo gastroscopy because of a chance of cure of an otherwise highly lethal disease. Okusa et al. found the prevalence of familial gastric carcinoma to be 10% of all gastric carcinoma. In the United States, the gastric carcinoma death rate has fallen from >30 per 100,000 in 1930 to ~5 per 100,000 in 1992 [1], and a decline also has been observed in Japan. In 1996, 22,800 diagnoses of stomach carcinoma are expected to be diagnosed in the United States [1]. If the familial rate were 10%, 2,280 kindreds could be identified, of which 80% (1,824) would be identified by the p53 test, whereas another 456 would be missed because of the 80% sensitivity rate of the test. Another 8,665 patients with gastric carcinoma would be identified erroneously as belonging to gastric carcinoma kindreds because of the 38% false positive rate. The total number of kindreds to be screened would then be 8,665 + 1,824 = 10,489.

Before considering a screening program based on the gastric carcinoma p53 test, confirmation of its relationship to familial gastric carcinoma is needed. Next, a study of the receiver operator characteristic of the test, i.e., the selection of an ideal cutoff point giving the best obtainable sensitivity to false positive ratio should be done. Relatives of patients with p53-positive gastric carcinoma could then be offered information on which they could base a decision on whether to accept screening for gastric carcinoma.

**John S. Meyer, MD**
Department of Pathology
St. Luke's Hospital
Chesterfield, Missouri 63017

## REFERENCES

1. Parker SL, Tong SB, Bolden S, Wingo PA: Cancer Statistics, 1996. CA 46:5–27, 1996.